

GERM CELL NUCLEAR FACTOR IS A TRANSCRIPTIONAL REPRESSOR ESSENTIAL FOR EMBRYONIC DEVELOPMENT

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1. ABSTRACT

GCNF is an orphan member of the nuclear receptor superfamily. The nuclear receptor superfamily is a large superfamily of transcription factors, the majority of which are designated as orphan receptors because their ligands and functions are currently unknown. GCNF (Germ Cell Nuclear Factor) is so named because of its restricted expression pattern in the adult. In the testis, GCNF is expressed only in the post meiotic round spermatids. Likewise in the ovary, GCNF's expression is restricted to the growing oocyte. To date nothing is known of GCNF's putative ligand; however, much is known about its physiological function through the use of gene targeting. Inactivation of the GCNF gene showed that it was essential for normal embryonic development. In addition to being expressed in the germ cells of the adult, it is expressed widely throughout the embryo after gastrulation. Significant strides have also been made in understanding GCNF's mechanism of action using molecular biology. The DNA binding properties of GCNF have been investigated and its response element identified. GCNF binds as a homodimer to a direct repeat element with zero nucleotides between the reiterated sequence AGGTCA. GCNF target genes have been identified that contain this DR0 element in their promoters. Such genes as Protamines 1 and 2 and Oct4 are regulated by GCNF through this element. GCNF has been shown to be a repressor of the protamine and Oct4 genes. GCNF's repression function has been shown to be mediated by interaction with the co-repressors N-CoR and SMRT in the absence of ligand. Our current efforts are to explore GCNF function in the adult germ cells using tissue specific gene targeting to specifically knock out the GCNF gene in oocytes and spermatogenic cells. In addition, efforts are being made to identify the endogenous ligand that regulates GCNF's transcriptional properties.

2. INTRODUCTION

One factor that has been identified as a possible regulator of spermatogenic specific genes is an orphan nuclear receptor called Germ Cell Nuclear Factor, or GCNF (1). GCNF is a member of the nuclear receptor superfamily. The nuclear receptor superfamily is a large superfamily of transcription factors, composed of 48 human members (2). The original members of the superfamily are the steroid receptors, which were initially identified by their ability to bind to the steroid hormones (3). Subsequent cloning of the steroid receptor genes, along with the genes for the receptors of thyroid hormone and vitamin D, revealed that they all share a similar structure. Nuclear receptors are characterized by the presence of distinct, separable domains: a highly variable N-terminal region, a type II zinc finger-containing DNA binding domain (DBD), a structurally flexible hinge region, a ligand binding domain (LBD), and the presence or absence of a C-terminal domain (4). Elucidation of this shared structure led to the discovery of related proteins by various methods of homology cloning and biochemistry (5). These receptors have been termed orphan nuclear receptors, since they were discovered without knowledge of their functions or activating ligands, if any. Therefore, studies of the molecular biology of these orphan receptors have been extensive in an effort to uncover their individual functions.

3. GCNF DNA BINDING PROPERTIES

GCNF is one of the more unusual orphan receptors in the nuclear receptor superfamily. While it has some properties in common with other orphan receptors, it also has unique properties that separate it from other nuclear receptors. Initial sequence analysis of GCNF was the first clue that GCNF fit into its own niche. The DBD of GCNF, which is the most highly conserved feature of the

superfamily, is only ~60% identical to RXR α and RAR α , with which it shares the most homology in this domain (1). Homology of the remainder of the GCNF sequence with other members decreases dramatically, with the highest degree of homology existing with COUP-TF I in the LBD (1). GCNF has therefore been tentatively classified into its own subfamily (6, 7). Closer analysis of the DBD sequence, however, shows that GCNF shares key residues with RAR and RXR in the P box, and with SF-1 in the A box (1). This is true for all known paralogs of GCNF (1, 8, 9, 10). The P box contains three amino acids which are critical for binding site recognition (11, 12). These residues (EGckG) convey binding to the AGGTCA half-site by GCNF. Most nuclear receptors that have this P box sequence heterodimerize with RXR. However, the similarity of GCNF's A box with that of SF-1, which binds as a monomer to an extended half-site (HS; T/GCAAGGTCA) indicated that GCNF may differ from other receptors in this respect. Indeed, electrophoretic mobility shift assay (EMSA) analysis by several groups has shown that DNA binding predictions based on the DBD sequence are correct (1, 8, 13, 14). GCNF has been found to bind as a homodimer to a direct repeat of the half-site AGGTCA, as predicted by its P box sequence, with the repeats separated by zero base pairs (DR0). Furthermore, a DR0 contains a SF-1 HS (aggTCAGGTCA), and GCNF can also bind this HS as a homodimer and monomer (1, 15), indicating that the A box of GCNF is involved in DNA binding. GCNF does not heterodimerize with RXR (16) and does not bind to DRs with spacings of one to six nucleotides (14). GCNF therefore has quite a restricted DNA binding specificity for an orphan nuclear receptor. In contrast, many orphan nuclear receptors have some flexibility in their ability to bind to direct repeats of different spacings such as COUP-TF (14, 17). While the spacing of the repeats is critical for GCNF binding, there is some flexibility within the consensus binding site, and the 5' sequence may increase the affinity of binding (13). This may have implications for GCNF responsive elements *in vivo*.

4. TRANSCRIPTIONAL PROPERTIES OF GCNF

Based on its ability to bind to a DR sequence as a homodimer, GCNF has been classified as a dimeric orphan receptor (18). Members of this class have been shown to be either activators or repressors of gene transcription (7, 18, 19), or both an activator or repressor in the case of COUP-TF (20). GCNF, in the presumed absence of ligand in transient transfection assays, acts as a repressor of both basal and ERR α 1-activated transcription (14, 21). In both cases, repression by GCNF is mediated by a DR0 sequence (14, 21). Repression by GCNF of ERR α 1-induced transcription is at least partly due to competitive DNA binding, since ERR α 1 binds to a similar sequence as GCNF (21). However, competition for binding is not the only mechanism by which GCNF can repress transcription. Recent work has shown that GCNF represses by interactions with co-repressors such as N-CoR and SMRT (21, 22), similar to other dimeric orphan receptors such as COUP-TF (23). Using the mammalian two-hybrid system, interactions between GCNF and N-CoR have been shown

to be dependent on helix 3 and helix 12 of the LBD (21). GCNF is therefore able to actively repress transcription, as well as compete for binding sites of activating factors.

5. DIMERIZATION STATUS OF GCNF

Recent work by Greschik *et al.* (15) has shown that homodimerization of GCNF is critical for its repressor function as well as binding to the DR0 sequence, and is dependent on three separate domains. One domain, comprised of both the T and A boxes at the C-terminal end of the DBD, also has been shown to be involved in DNA interactions. A construct comprised of GCNF DBD and the SF-1 TA domains can not bind to a DR0 sequence as a homodimer. In contrast, a GCNF DBD/GCNF TA construct binds to a DR0 element as both a homodimer and a monomer. However, substitution of the SF-1 TA domains for the corresponding GCNF domains in the full length GCNF (GCNF/SF-1 TA) lowers affinity of GCNF for the DR0 sequence, but does not abrogate homodimer formation, and only binds the HS sequence as a monomer, indicating that the GCNF TA domains are involved in proper GCNF homodimerization on the DR0 and HS sequences (15). Another domain that contributes to dimerization of GCNF is helix 3 of the LBD. Deletion or point mutation of helix 3 disrupts dimerization of a truncated GCNF on the HS sequence. Replacement of the GCNF LBD with the hRXR α LBD causes loss of homodimerization on an HS sequence. Returning helix 3 of GCNF to this chimera (*i.e.*, GCNF N-terminus through hinge region, hRXR α helices 1-2, GCNF helix 3, hRXR α helices 4-12) restores the ability to homodimerize. This dimerization property is unique to GCNF, since in other receptors, dimerization has been shown to be dependent either on helices 9-10 (24, 25, 26, 27) or helices 5-7 (28). Furthermore, in RXR and thyroid hormone receptor (TR), helix 3 has been shown to be important for binding co-repressors (29) or co-activators (30, 31). GCNF is the first receptor observed to use helix 3 as a dimerization interface. Whether helix 3 is important in GCNF interactions with co-factors remains to be determined. The third dimerization domain for GCNF is in helix 12 of the LBD. This is yet another unique feature of GCNF, because helix 12 in other receptors has been shown to be important for ligand binding, but not for dimerization (15). Although it has yet to be determined if helix 12 in GCNF is important for ligand binding, deletion or point mutation of helix 12 lowers its affinity for DNA binding to a DR0, and disrupts dimer binding on the HS sequence. Helix 12 mutants however, are still able to bind the HS element as a monomer (15). GCNF's mechanism of dimerization, involving the TA boxes, helix 3, and helix 12, is unique among nuclear receptors, and may allude to a novel transcriptional regulatory mechanism employed by GCNF.

Dimerization of GCNF is important, not only because of higher affinity DNA binding, but also because GCNF's repressor function is dependent on its dimer status. Mutants of helix 12 of the LBD which do not bind as homodimers to the HS, are no longer able to repress basal transcription of a reporter gene linked to multiple copies of

the HS (15). Furthermore, the lower affinity of helix 12 mutants for the DR0 sequence also affects its repressor function (15). Presumably, the observed effects are due to disruption of co-repressor interactions, dependent on helix 12 (21). Disruption of dimerization due to mutations in helix 3 also may affect repression function of GCNF, since mutation in helix 3 also disrupts co-repressor interactions (21). Effects of the other dimerization interface on repression, the TA box of the LBD, have not been tested in functional assays, but would presumably show lower repressor activity due to decreased dimerization and affinity for DNA binding.

While GCNF has not yet been shown to activate transcription, the mutations in helix 12 of GCNF, which affect dimerization appear to involve conformational changes of the receptor similar to those involving helix 12 in other receptors upon ligand binding. These conformational changes dissociate receptors from co-repressors and allow for interface with co-activators (15). Indeed, even though GCNF does not contain an AF-2 domain in its LBD ($\Phi \Phi X(E/D)\Phi \Phi$) (32), point mutation of helix 12, the location of the AF-2 in other receptors, causes a loss of repressor function in GCNF (15). Induction of conformational changes that affect the association state of co-repressors with GCNF, by some as yet unknown stimulus, may uncover domains that interact with co-activators, conferring on GCNF an ability to activate transcription. Similarly, structural predictions based on primary amino acid sequence suggest that the first 11 helices of the LBD in GCNF, form an α -helical sandwich similar to those observed in other receptors (15). Coupled with the intrinsic importance of GCNF's helix 12, this predicted structure strongly suggests that GCNF undergoes the conformational changes necessary to convert it from a repressor to an activator. Furthermore, it suggests that GCNF may contain a ligand-binding pocket similar to other receptors, and may therefore have the ability to bind a ligand (26). Thus, the mechanism of the novel signaling pathway mediated by GCNF may be similar to that of TR, in that it represses the expression of genes in the absence of a signal and may activate them upon binding ligand. Alternatively, the absence of a canonical AF2 region in GCNF indicates that the putative GCNF ligand may act through relieving repression of target genes as opposed to truly transactivating their expression.

6. GCNF TARGET GENES

Even though activation of genes by GCNF is still in the speculative stages, there is now evidence that GCNF can repress genes *in vivo*. Induction of GCNF in P19 cells by retinoic acid (RA) treatment had previously been observed (33), which inversely correlated with a reduction in Oct4 expression upon treatment of P19 cells with RA (34). EMSA analysis of the Oct4 promoter identified GCNF as part of a protein complex which binds to an imperfect DR0 located there (22). Transient transfection of a GCNF expression plasmid into P19 cells resulted in down-regulation of Oct4 expression, in a manner similar to RA treatment, and mutation analysis of the DR0 in the promoter indicated that this down-regulation by GCNF was

mediated by the DR0 (22). Furthermore, mouse embryos lacking a functional GCNF fail to repress the Oct4 gene (22) after gastrulation. Normal embryonic expression of Oct4 is quickly down-regulated in the epiblast in an anterior to posterior manner, and by E8.25 is restricted to primordial germ cells. In GCNF homozygous null mutant mice, however, Oct4 continues to be expressed more widely than just the primordial germ cells in the embryo. GCNF therefore is necessary for appropriate repression of Oct4 *in vivo*, and Oct4 becomes the first well-established target gene of GCNF.

7. EMBRYONIC FUNCTION OF GCNF

Although Oct4 is the only well-established target gene of GCNF regulation, homologous recombination experiments in mice have determined GCNF is necessary for proper embryo formation, and therefore must be regulating a wider array of essential genes (35). As expected from the widespread expression pattern in the embryo, multiple tissue systems are affected. The major defect in mice is a posterior truncation after formation of approximately 13 somites, followed by death of the embryo at E10.5 (35). Additional defects include failure of the neural tube to close, ectopic tailbud formation, a distended pericardium, and extra-embryonic tissue defects, as well as a turning defect (35). Similar studies in frogs, involving over expression of GCNF and the use of a dominant negative GCNF, also show anteroposterior defects (36). GCNF therefore clearly regulates genes important for embryogenesis, presumably by a failure to silence its target genes, as suggested by all available functional data. Furthermore, the function of GCNF as an important factor in embryogenesis is evolutionarily conserved from amphibians to mammals. Identification of additional target genes will further elucidate both the function of GCNF and the mechanisms of appropriate patterning during embryogenesis.

8. THE ROLE OF GCNF IN SPERMATOGENESIS AND OOGENESIS

Although the role of GCNF in the embryo is slowly being uncovered, its role in adult animals remains something of a mystery. Originally cloned from a mouse heart cDNA library by low-stringency hybridization screening with the DBD of mNUC1, Northern analysis of adult mouse tissues subsequently showed that GCNF is most highly expressed in the testis, with limited expression in the lung and kidney (1). *In situ* hybridization analysis of the testis showed that GCNF is expressed exclusively in the spermatogenic cells. Similar analysis of the ovary showed expression exclusively in the oocyte, hence the original name (1). To date, most studies of GCNF in adult animals have been limited to the testis and ovary. Expression of mouse GCNF in the testis is limited to post meiotic round spermatids, with the highest levels of message observed at stages VII-VIII (37). A similar pattern is observed in rat testes (38). However, one report in mice claims additional expression of GCNF in late-stage primary spermatocytes (39), which may be due to strain differences. A similar observation of expression pattern is observed in the human,

where the highest levels of GCNF mRNA appear to be in the late-stage primary spermatocytes (40). Regardless, localization of GCNF to spermatogenic cells suggests that it plays a role in regulating genes important for the terminal differentiation of spermatogenesis. Multiple genes in the mouse, which are expressed during terminal differentiation of spermatogenic cells, known as spermiogenesis, have been identified which contain DR0 sequences in their promoters. Genes expressed during spermatogenesis include, Oct4, the testis-specific form of ACE, endozepine-like protein (ELP), the protamine genes, as well as others (41). The protamine genes, which are critical to the proper differentiation of sperm in both mice (42, 43) and humans (44, 45), are small, highly basic proteins that replace the histones in the chromatin structure of the developing sperm. Binding studies *in vitro* have shown that GCNF can bind to the DR0 sequences in both the mouse protamine 1 and 2 gene promoters (46). More recently, GCNF has been shown to bind the DR0 sequence in the ELP promoter (41). Ongoing studies in spermatogenic cell specific GCNF mutant mice will elucidate whether GCNF also represses transcription of the protamine genes and other genes in spermatogenesis.

Less is known about GCNF in oocyte maturation. However, it is known that GCNF expression is restricted to the developing oocytes, appearing at the primary follicle stage, and persisting through the pre-ovulatory follicle stage (1, 37). GCNF is not expressed in primordial follicles (37). GCNF therefore may be involved in follicle growth, oocyte maturation, or more directly in regulating genes involved in meiosis. Identification of target genes in oocytes and generation of an oocyte specific GCNF mutant mouse will be necessary to determine if GCNF plays a similar role in oocytes as it does in spermatogenesis.

9. PERSPECTIVE

The role of GCNF at this point appears to be as a repressor of transcription in such diverse processes as embryonic development, neurulation, placental development, spermatogenesis, and oogenesis during which GCNF is expressed. GCNF obviously does not exist in a vacuum, and must be an important element of an as yet unidentified signaling pathway. Identification of GCNF target genes is giving investigators some insight into the downstream actions of GCNF, but upstream effectors of GCNF are still mostly unknown. Searches for activating ligands of GCNF, which would be the most obvious upstream effector, have so far been unsuccessful. One upstream effector of GCNF appears to be RA. Treatment of embryonal carcinoma cell lines with RA affects the expression of GCNF in these cells (33, 47); however, there are differing reports as to what effect RA has on GCNF expression. Endogenous GCNF expression in undifferentiated F9, PCC4.aza1R, OC15S1 and NT2/D1 cells has been reported to be down-regulated upon RA-induced differentiation (47). In contrast, a different research group shows that undifferentiated F9, P19, and PCC7-Mz1 cells do not express GCNF, and that RA treatment up-regulates GCNF expression in these cells (33). Regulation of GCNF therefore appears to depend on the

cell type and possibly the cell culture conditions, and it remains to be seen if RA affects GCNF expression through a direct or indirect pathway. Cloning of the promoter for GCNF will allow closer analysis of RA's effects on GCNF, and give direction as to other upstream factors.

The regulatory function of GCNF has now been partly elucidated. It is now established that at in some instances, GCNF fits into the category of a repressor of transcription. However, some of the properties of GCNF repression are novel, including its use of different dimerization surfaces, its response element, and its pattern of expression. These properties set it apart from other nuclear receptors, supporting the classification to its own subfamily, which was initially based on sequence comparisons. The uniqueness of GCNF should give further insight into mechanisms of nuclear receptor function and transcriptional regulation in general. Further studies of GCNF function will also enlighten us about how it affects the processes of embryogenesis and gametogenesis, as well as increase our general knowledge of these two processes. Thus, the role of GCNF is not just as a repressor of transcription, but also as a model of transcriptional regulation in several important biological processes.

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